Reference: *Biol. Bull.* **180**: 485–488. (June, 1991)

New Calcitonin Isolated from the Ray, *Dasyatis akajei*

Y. TAKEI[1]*, A. TAKAHASHI[2], T. X. WATANABE[3], K. NAKAJIMA[3], S. SAKAKIBARA[3], Y. SASAYAMA[4], N. SUZUKI[4], AND C. OGURO[4]

Departments of [1]Physiology and [2]Molecular Biology, Kitasato University School of Medicine, Sagamihara, Kanagawa 228, [3]Peptide Institute Inc., Protein Research Foundation, Minoh, Osaka 562, and [4]Department of Biology, Faculty of Science, Toyama University, Toyama 930, Japan

Abstract. Calcitonin causes hypocalcemia by inhibiting the resorption of calcium from the bone in mammals. Calcitonin has now been isolated from the ultimobranchial gland of a cartilaginous fish, the ray (*Dasyatis akajei*), and its amino acid has been determined to be H-Cys-Thr-Ser-Leu-Ser-Thr-Cys-Val-Val-Gly-Lys-Ser-Gln-Gln-Leu-His-Lys-Leu-Gln-Asn-Ile-Gln-Arg-Thr-Asp-Val-Gly-Ala-Ala-Thr-Pro-NH_2. Although its basic structure is well conserved, the amino acid sequence of ray calcitonin is considerably different from that of other calcitonins sequenced to date. Because the ray lacks calcified bones, an examination of the effect of calcitonin in this fish may elucidate a new role for calcitonin in vertebrates.

Introduction

Calcitonins were first isolated from the thyroid glands of mammals, and amino acid sequences have now been determined in five species (Neher *et al.*, 1968; Potts *et al.*, 1968, 1971; Brewer and Ronan, 1969; Raulais *et al.*, 1976). Although calcitonin-like immunoreactivity was also identified in the ultimobranchial glands of all classes of non-mammalian vertebrates (Van Noorden and Pearse, 1971; Tisserand-Jochem *et al.*, 1977; Sasayama *et al.*, 1984; Treilhou-Lahille *et al.*, 1984), structures have been determined for only one species of bird and two teleost fishes (Niall *et al.*, 1969; Noda and Narita, 1976; Homma *et al.*, 1986). The mammalian calcitonins fall into two groups according to the homology of their amino acid sequences, and the difference between the amino acid sequences of these two mammalian groups is greater than that between the bird and the teleosts (Fig. 1). As for function, non-mammalian calcitonins have a much greater hypocalcemic effect in the rat than do the mammalian calcitonins (Homma *et al.*, 1986).

Recently, immunoreactive calcitonin was demonstrated in the ray, *Dasyatis akajei* (Sasayama *et al.*, 1984). Because the osteocytes are the principal site of action of calcitonin in mammals (Friedman and Raisz, 1965), a new action of calcitonin should be expected in this cartilaginous fish. Indeed, mammalian calcitonin has been shown to lack a hypocalcemic effect in some non-mammalian species (Pang *et al.*, 1980). Thus, the roles of calcitonin in the cartilaginous fish may provide a new insight into the fundamental actions of calcitonin common to all vertebrates. As the essential step toward discovering such roles, an attempt was made to isolate calcitonin from the ray and to determine its amino acid sequence. The molecular structure of calcitonin in this phylogenetically primitive fish may provide new evidence for the evolution of the calcitonin molecule in vertebrate phylogeny.

Materials and Methods

The rays (*Dasyatis akajei*) were caught in Toyama Bay and anesthetized with 1/3,000 (v/v) of tricaine methanesulfonate (Sigma) in seawater. The ultimobranchial glands were resected under a dissecting microscope, immediately frozen, and kept at −50°C until used.

Calcitonin was extracted from ray ultimobranchial glands and purified as follows. Two hundred deep-frozen glands (2.2 g) were pulverized in a stainless-steel crusher with a hammer, immediately boiled for 5 min with 7 volumes of water, acidified with acetic acid to make a final concentration of 1 *M*, and homogenized in a Polytron homogenizer for 90 s at 4°C at maximum speed (Takei *et al.*, 1989). The homogenate was centrifuged at 25,000 × *g* for 30 min at 4°C, and the high molecular weight proteins and lipids were removed from the supernatant

Received 22 February 1991; accepted 27 March 1991.

* To whom correspondence should be sent.

```
Fowl CT      CASLSTCVLGKLSQELHKLQTYPRTDVGAGTP-NH2
Eel CT       CSNLSTCVLGKLSQELHKLQTYPRTDVGAGTP-NH2
Salmon CT    CSNLSTCVLGKLSQELHKLQTYPRTNTGSGTP-NH2

Rat CT       CGNLSTCMLGTYTQDLNKFHTFPQTSIGVGAP-NH2
Human CT     CGNLSTCMLGTYTQDFNKFHTFPQTAIGVGAP-NH2

Porcine CT   CSNLSTCVLSAYWRNLNNFHRFSGMGFGPETP-NH2
Bovine CT    CSNLSTCVLSAYWKDLNNYHRFSGMGFGPETP-NH2
Ovine CT     CSNLSTCVLSAYWKDLNNYHRYSGMGFGPETP-NH2
```

Figure 1. Amino acid sequences of the mammalian and non-mammalian calcitonins (CTs) that have been sequenced to date. The identical amino acids within the same group are boxed.

with 67% and 98.5% acetone, respectively, at 4°C. The extract was then subjected to reverse-phase high performance liquid chromatography (HPLC) on an ODS-120T column (4.6 × 250 mm; Tosoh, Tokyo) with a linear gradient elution from 20% to 80% CH_3CN in 0.1% trifluoroacetic acid (pH 2.0), and each fraction was examined for the presence of immunoreactive calcitonin by immunoblotting. Each immunoreactive fraction was finally purified on the same column with a solvent of a different pH (ammonium acetate buffer, pH 4.6).

The fractions were lyophilized and a small portion of each, or synthetic salmon calcitonin (1 ng–1 μg), was dissolved in 10 μl of a mixture of 0.1 *M* Na_2CO_3 (pH 9.5) and methanol (4:1, v/v), and blotted onto an Immobilin PVDF transfer membrane (Millipore Co. Ltd., Tokyo). The membrane was soaked in 100% methanol for 3 s, and washed 3 times in 10 m*M* phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20 (PBST) for 5 min. The membrane was washed twice more in PBST containing 1% normal goat serum, and then three times again in PBST. The membrane was then incubated with an antiserum raised against salmon calcitonin (Sasayama *et al.*, 1989) (1/40,000 dilution) for 2 h at room temperature. The unbound antiserum was removed by three washes in PBST, and the membrane was immunostained with a Vectastain ABC kit (Vector Laboratories, California) according to the protocol included with the kit.

A portion of purified ray calcitonin was subjected to reduction and S-carboxymethylation, as reported previously (Takei *et al.*, 1989), and further purified by reverse phase HPLC. The amino acid sequence of the purified peptide was determined with a protein sequencer (Applied Biosystems, Model 470A/120A). The sequence thus determined was verified by the amino acid analysis (15), and by coelution of the purified and synthetic peptides in reverse-phase HPLC with two different solvent systems (Takei *et al.*, 1990). The ray calcitonin was synthesized by a peptide synthesizer (Applied Biosystems, Model 430A) as reported previously (Takei *et al.*, 1989). The correct sequence of the synthetic peptide was confirmed by amino acid analysis, and by the sequencer.

Results

At first, 1/10 of the crude acid extract of ray ultimobranchial glands was subjected to reverse phase HPLC, and several fractions showed immunoreactivity to the antibody raised against salmon calcitonin (Fig. 2). Each positive fraction was further chromatographed with a solvent of different pH, and only one immunoreactive peak was detected from one of the positive fractions (Fig. 3). No immunoreactive material was recovered from the other fractions. The height of the peak was equivalent to 12.2 nmoles of salmon calcitonin. Thus, the ultimobranchial gland of the ray contains at least 60 nmoles/g tissue of calcitonin. The amino acid sequence of the purified material was determined by sequencer (Fig. 4).

The ray calcitonin was also purified from the remaining 9/10 of the crude extract. This material was then reduced and S-carboxymethylated, and 1/10 of the carboxymethylated peptide was subjected to amino acid analysis to verify the sequence. The ray calcitonin was composed of

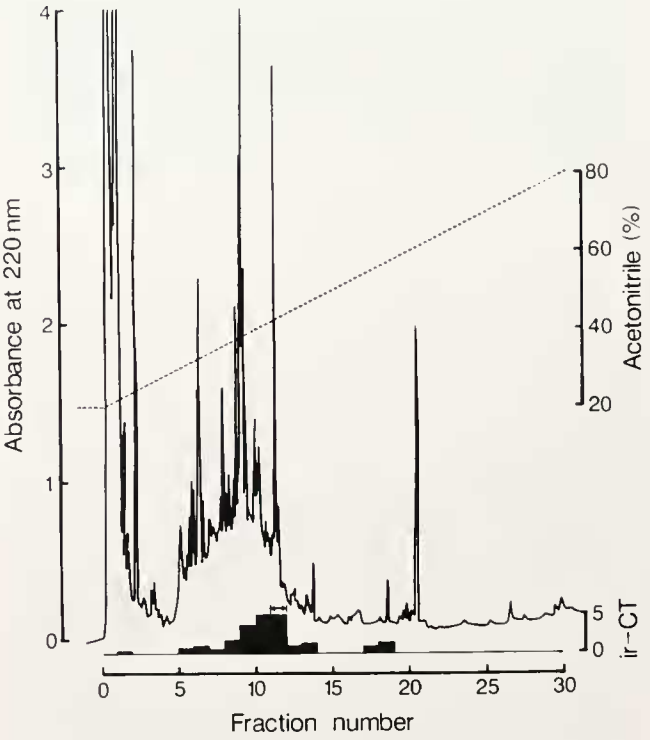


Figure 2. Reverse phase HPLC on an ODS-120T column. Sample, crude acid extract of ultimobranchial glands of the ray; flow rate, 1 ml/min; fraction size, 2 ml/tube. Solvent system: linear-gradient elution from 20 to 80% CH_3CN in 0.1% trifluoroacetic acid for 60 min. The immunoreactive calcitonin (ir-CT) was quantified by scores from 0 to 5. Arrows indicate the fraction within which ray calcitonin was eluted.

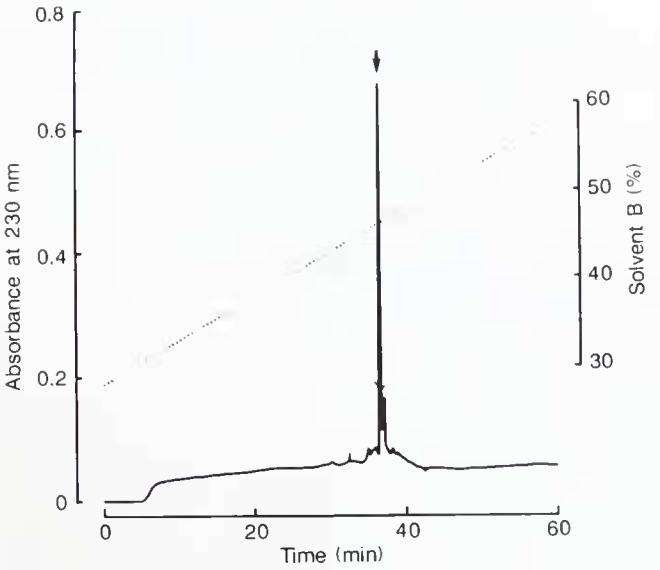


Figure 3. Reverse phase HPLC on an ODS-120T column. Sample, fraction 12 in Figure 2; flow rate, 1 ml/min. Solvent system: linear-gradient elution from solvent A (H_2O : CH_3CN : 1 M NH_4OAc, pH 4.6 = 72 : 8 : 1, v/v) to B (H_2O : CH_3CN : 1 M NH_4OAc, pH 4.6 = 25 : 100 : 1, v/v) for 40 min. Fraction was collected at each peak. Immunoreactivity appeared only in the peak marked by the arrow.

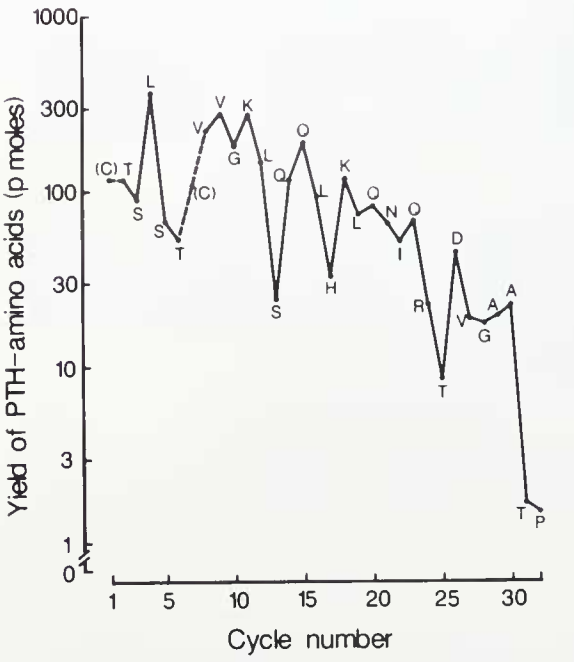
32 amino acid residues: Asp, 2.0 (2); Glu, 4.6 (4); CM-Cys, 1.3 (2); Ser, 3.0 (3); Gly, 2.2 (2); His, 1.0 (1); Arg, 1.1 (1); Thr, 4.2 (4); Ala, 2.2 (2); Pro, 1.1 (1), Val, 2.4 (3), Ile, 1.0 (1), Leu, 4.2 (4), Lys, 2.0 (2); the numbers in parentheses were deduced from the sequencing. Since the cysteine residue was undetectable without carboxymethylation in a sequencer, the carboxymethylated material was also subjected to the sequencer and the presence of cysteine residues at the first and the seventh position was confirmed. The amidation of the proline residue at the C-terminus was determined by co-chromatography with a synthetic peptide in reverse-phase HPLC.

Discussion

In this study, a large amount of calcitonin (more than 60 nmoles/g tissue) has been detected in the ultimobranchial glands of the ray (*Dasyatis akajei*), and sequenced. This is the first elasmobranch calcitonin to have been characterized.

In mammals, calcitonin is a hypocalcemic hormone that inhibits the reabsorption of calcium from the bone (Friedman and Raisz, 1965). Among non-mammals, calcitonin-like immunoreactivity has been detected in selected species from all classes, and calcitonin has been isolated in a bird and teleosts (Niall *et al.*, 1969; Van Noorden and Pearse, 1971; Noda and Narita, 1976; Tisserand-Jochem *et al.*, 1977; Sasayama *et al.*, 1984; Treilhou-Lahille *et al.*, 1984; Homma *et al.*, 1986), but the physiological roles of the hormone in these species are not fully understood. Because a hypocalcemic effect is not common in non-mammalian species (Pang *et al.*, 1980), cartilaginous fishes, which appear to have large amounts of calcitonin, may be good material with which to investigate those roles of the hormone that have been retained throughout the vertebrates. The use, in such studies, of the native hormone, now available, is important because mammalian hormones often have little biological effect in fishes (Takei *et al.*, 1989, 1990).

The calcitonins sequenced to date can be classified into three groups according to their structural similarity (Fig. 1). The sequence homology among the calcitonins within each group is 88–94% (Table 1). Ray calcitonin is apparently more homologous to non-mammalian calcitonins, but the homology is less than that within any of the groups. In particular, the calcitonins from fowl and ray are more similar than the two types of mammalian calcitonins. Non-mammalian calcitonins generally have greater hypocalcemic effects in the rat than do mammalian calcitonins (Homma *et al.*, 1986). Indeed, our preliminary



Ray CT CTSLSTCVVGKLSQQLHKLQNIQRTDVGAATP–NH_2

Figure 4. Automatic sequencer analysis of the purified peak of ray calcitonin immunoreactivity shown in Figure 3. The yield of phenylthiohydantoin-derivitized (PTH) amino acid is plotted for each cycle of Edman degradation. The cystine residues (C) at cycles 1 and 7 were not determined in this analysis, which was carried out without prior carboxymethylation (see Results). The complete amino acid sequence, finally verified by amino acid analysis and by co-chromatography with synthetic peptide, is set out below the plot.

Table I

*The sequence homology of amino acids (above *) and of nucleotides (below *) between two calcitonins from different species*

	Ray	Fowl	Eel	Salmon	Rat	Human	Pig	Ox	Sheep
Ray	*	78	75	66	38	34	31	31	31
Fowl	–	*	94	88	50	47	44	44	41
Eel	–	–	*	94	53	50	41	41	44
Salmon	–	74	–	*	59	56	41	44	41
Rat	–	66	–	60	*	94	47	47	44
Human	–	68	–	60	91	*	44	44	41
Pig	–	–	–	–	–	–	*	91	88
Ox	–	–	–	–	–	–	–	*	94
Sheep	–	–	–	–	–	–	–	–	*

Numbers are homologies expressed in terms of percentage. –; not examined. For nucleotide sequences, see Craig *et al.*, 1982; Rosenfeld *et al.*, 1984; Lasmoles *et al.*, 1985; Poeschl *et al.*, 1987.

results show that when rats are injected with 1 pmol of ray calcitonin, the plasma calcium concentration decreases by 15.3 ± 1.5% after 30 min, whereas injection with the same dose of human and porcine calcitonin causes a decrease of 9.8 ± 1.2% and 3.5 ± 0.7%, respectively (n = 10 in each case). Thus, ray calcitonin is apparently more hypocalcemic in the rat than mammalian calcitonins. Ray calcitonin may have a clinical application, as is the case for eel calcitonin (Orimo, 1979).

Acknowledgments

The authors are grateful to Dr. David A. Price of The Whitney Laboratory, University of Florida, for the critical reading of this manuscript. We also thank Miss S. Nishida for artwork. This investigation is supported in part by the Terumo Foundation.

Literature Cited

Brewer, H. B., Jr., and R. Ronan. 1969. Amino acid sequence of bovine calcitonin. *Biochemistry* **63:** 940–947.

Craig, R. K., L. Hall, M. R. Edbrooke, J. Allison, and I. MacIntyre. 1982. Partial nucleotide sequence of human calcitonin precursor mRNA identifies flanking cryptic peptides. *Nature* **295:** 345–347.

Friedman, J., and L. G. Raisz. 1965. Thyrocalcitonin: inhibition of bone resorption in tissue culture. *Science* **150:** 1465–1467.

Homma, T., M. Watanabe, S. Hirose, A. Kanai, K. Kangawa, and H. Matsuo. 1986. Isolation and determination of amino acid sequence of chicken calcitonin I from chicken ultimobranchial glands. *J. Biochem.* **100:** 459–467.

Lasmoles, F., A. Jullienne, F. Day, S. Minvielle, G. Milhaud, and M. S. Moukhtar. 1985. Elucidation of the nucleotide sequence of chicken calcitonin mRNA: direct evidence for the expression of a lower vertebrate calcitonin-like gene. *EMBO J.* **4:** 2603–2607.

Neher, R., B. Riniker, W. Rittel, and H. Zuber. 1968. Menschliches calcitonin. III. Struktur von calcitonin M und D. *Helv. Chim. Acta* **51:** 1900–1905.

Niall, H. D., H. T. Keutmann, D. H. Copp, and J. T. Potts, Jr. 1969. Amino acid sequence of salmon ultimobranchial calcitonin. *Proc. Natl. Acad. Sci. USA* **64:** 771–778.

Noda, T., and K. Narita. 1976. Amino acid sequence of eel calcitonin. *J. Biochem.* **79:** 353–359.

Orimo, H. 1979. Clinical application of calcium-regulating hormone. *Clin. Endocrinol.* **28:** 269–274.

Pang, P. K. T., A. D. Kenny, and C. Oguro. 1980. Evolution of endocrine control of calcium regulation. Pp. 323–356 in *Evolution of Vertebrate Endocrine Systems*, P. K. T. Pang, and A. Epple, eds. Texas Tech. Press, Lubbock.

Poeschl, E., I. Lindley, E. Hofer, J. M. Seifert, W. Brunowsky, and J. Besemer. 1987. The structure of procalcitonin of the salmon as deduced from its cDNA sequence. *FEBS Lett.* **226:** 96–100.

Potts, J. T., Jr., H. D. Niall, H. T. Keutmann, and R. B. Brewer, Jr., and L. J. Deftos. 1968. The amino acid sequence of porcine thyrocalcitonin. *Proc. Natl. Acad. Sci. USA* **59:** 1321–1328.

Potts, J. T., Jr., H. D. Niall, H. T. Keutmann, and R. M. Lequin. 1971. Chemistry of calcitonin; species variation plus, structure-activity relations and pharmacologic implications. Pp. 121–127 in *Calcium, Parathyroid Hormone and the Calcitonin*, R. V. Talmage and P. L. Munson, eds. Excerpta Medica, Amsterdam.

Raulais, D., J. Hagaman, D. A. Ontjes, R. L. Lundblad, and H. S. Kingdon. 1976. The complete amino-acid sequence of rat thyrocalcitonin. *Eur. J. Biochem.* **64:** 607–611.

Rosenfeld, M. G., S. G. Amara, and R. M. Evans. 1984. Alternative RNA processing events as a critical developmental regulatory strategy in nonendocrine gene expression. *Biochem. Soc. Symp.* **49:** 27–44.

Sasayama, Y., C. Oguro, R. Yui, and A. Kanbegawa. 1984. Immunohistochemical demonstration of calcitonin in ultimobranchial glands of some lower vertebrates. *Zool. Sci.* **1:** 755–758.

Sasayama, Y., K. Matsuda, C. Oguro, and A. Kambegawa. 1989. Immunohistochemical study of the ultimobranchial gland of chum salmon fry. *Zool. Sci.* **6:** 607–610.

Takei, Y., A. Takahashi, T. X. Watanabe, K. Nakajima, and S. Sakakibara. 1989. Amino acid sequence and relative biological activity of eel atrial natriuretic peptide. *Biochem. Biophys. Res. Commun.* **164:** 537–543.

Takei, Y., A. Takahashi, T. X. Watanabe, K. Nakajima, S. Sakakibara, T. Takao, and Y. Shimonishi. 1990. Amino acid sequence and relative biological activity of a natriuretic peptide isolated from eel brain. *Biochem. Biophys. Res. Commun.* **170:** 883–891.

Tisserand-Jochem, E. M., A. Eyquem, J. Peignoux-Deville, and C. Calmettes. 1977. Calcitonine du corps ultimobranchial de' Anguilles (*Anguilla anguilla* L.): localisation cytologique par immunofluorescence indirecte a l'aide d'un anticorps humain anti-calcitonine de Saumon. *C. R. Acad. Sci. Ser. D* **258:** 81–84.

Treilhou-Lahille, F., A. Jullienne, M. Aziz, A. Beaumont, and M. S. Moukhtar. 1984. Ultrastructural localization of immunoreactive calcitonin in the two cell types of the ultimobranchial gland of the common toad (*Bufo bufo* L.). *Gen. Comp. Endocrinol.* **53:** 241–251.

Van Noorden, S., and A. G. E. Pearse. 1971. Immunofluorescent localization of calcitonin in the ultimobranchial gland of *Rana temporaria* and *Rana pipiens*. *Histochemie* **26:** 95–97.